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The Phagocytosis of Red Blood-Cells.

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It has been shown by Savtschenko,* that when an animal has received injections of red blood-cells, its serum (inactivated by heating) causes the appearance of phagocytosis *in vitro* when leucocytes and red blood-cells of the kind used for injection are added, and the whole maintained at the temperature of the body. This action is attributed by Savtschenko to the action on the red blood-cells of amboceptor (immunesine, fixateur) contained in the serum employed.

In order to obtain further information respecting the factors which determine phagocytosis *in vitro* of red blood-cells, a comparative examination of the action of sera of animals nearly related to, and widely separated from, those supplying the erythrocytes used for injection was undertaken. This investigation confirmed the above observation, that leucocytes placed in the inactivated serum of injected animals ingested red blood-cells of the kind used for injection. In the course of this investigation, however, it was found that phagocytosis could be brought about by the serum of the injected animals when the serum was free from amboceptor, for the red blood-cells injected, as the following experiment shows:—

Experiment 1.—Into the abdominal cavity of a dove, the red blood-cells

* "Du Rôle des Immunesines (Fixateurs) dans la Phagocytose," 'Annales de l'Institut Pasteur,' 1902, vol. 16, p. 106.

from the following amounts of hen's blood were injected: on the 1st day, 6 c.c.; on the 6th day, 6 c.c.; on the 15th day, 9 c.c.; on the 38th day, 5 c.c. On the 45th day the animal was killed. Some leucocytes from a dove, together with erythrocytes from a hen, were added to a small quantity of the serum of the injected animal. After the lapse of an hour, at 37° C., about half the leucocytes present contained one or two, or rarely more, red blood-cells. 0.5 c.c. of the serum of the injected animal was added to 1 c.c. of a 2.5 per cent. suspension in 0.85 per cent. sodium chloride solution of hen's erythrocytes, and the volume of the mixture then made up to 2 c.c. by the further addition of 0.5 c.c. of the salt solution. At the end of 5 minutes agglutination was complete; at the end of 24 hours, at 37° C., no trace of hæmolysis was perceptible. Smaller amounts of serum were also employed, with negative results, as far as the production of hæmolysis is concerned.

It follows from this experiment, therefore, that the presence of amboceptor is not causally related to the development of phagocytosis, since phagocytosis can occur in the absence of amboceptor.

It was also found that, in most of the experiments made, agglutinin was present, and the enquiry presented itself whether agglutinin was essential to phagocytosis. It was found, however, that in some cases phagocytosis made its appearance in sera which possessed no agglutinating power for the red blood-cells employed. Among experiments illustrating this point are the following:—

Experiment 2.—Into the abdominal cavity of a rabbit, the red blood-cells obtained from the following amounts of calf's blood were injected: on the 1st day, 20 c.c.; on the 6th day, 20 c.c.; on the 17th day, 80 c.c. On the 24th day blood was taken from the animal and serum obtained. To a small amount of this serum, after inactivation by heating to 58° C. for 30 minutes, leucocytes from the rabbit and erythrocytes from the calf were added. At the end of half an hour at 37° C., well-marked phagocytosis had occurred, about one-third of the leucocytes present having taken up one to three red blood-cells. To test the agglutinating power of the serum, which before inactivation was strongly hæmolytic, the following tests were made: to four test-tubes, each of which contained 1 c.c. of a 2.5 per cent. suspension (in 0.85 per cent. NaCl solution), 0.5 c.c., 0.25 c.c., 0.1 c.c., and 0.05 c.c. of inactivated serum respectively were added, and the bulk of fluid in each test-tube made up to 2 c.c. At the end of 24 hours, at a temperature of 37°, no agglutination had occurred in any of the tubes.

Experiment 3.—Into the abdominal cavity of a guinea-pig the red blood-cells obtained from the following amounts of calf's blood were injected: on the 1st day, 3 c.c.; on the 3rd day, 10 c.c.; on the 14th day, 7 c.c.

On the 30th day blood was taken from the animal and serum obtained. To a small amount of this serum (which when active was strongly hæmolytic), after inactivation, leucocytes from the guinea-pig and erythrocytes from the calf were added. At the end of half an hour, at 37° C., one-quarter of the leucocytes present were found to contain one to three or more red blood-cells. On testing the agglutinating power of the serum, as in the preceding experiment, no agglutination was observed in any of the test-tubes, even at the end of 24 hours.

Experiment 4.—Into the abdominal cavity of a guinea-pig the red blood-cells obtained from the following amounts of goat's blood were injected: on the 1st day, 10 c.c.; on the 6th day, 12 c.c.; on the 31st day, 12 c.c.; on the 41st day, 15 c.c. On the 49th day blood was taken from the animal and serum obtained. To a small amount of this serum, after inactivation, leucocytes from the rabbit and erythrocytes from the goat were added. At the end of half an hour at 37° C., well-marked phagocytosis had occurred, about one-third of the leucocytes containing red blood-cells; at the end of an hour more than three-quarters of the leucocytes had taken up red cells, which were observed to be more or less pale. On testing the agglutinating power of the serum, as in Experiment 3, no agglutination was observed in any of the test-tubes, even at the end of 24 hours.

From these experiments it follows that the presence of agglutinin is not a necessary factor in the production of phagocytosis.

The former of the above conclusions is confirmed by the behaviour of normal rabbit serum. This has a marked hæmolytic action on the red blood-cells of the goat and guinea-pig. Nevertheless in the combination: *inactivated normal rabbit serum + erythrocytes of goat + leucocytes of rabbit*, and *inactivated normal rabbit serum + erythrocytes of guinea-pig + leucocytes of rabbit* phagocytosis *in vitro* does not occur, so that in normal serum the presence of amboceptor is insufficient to bring about phagocytosis. None of the normal sera employed possessed a powerful agglutinating action upon any of the red blood-cells employed, so that a similar demonstration of the inefficiency of agglutinin cannot be given.

Since the sera of animals into which red blood-cells have been injected is capable of bringing about phagocytosis when amboceptor and agglutinin are absent, it follows that this property of such sera is due to some special substance which is not amboceptor or agglutinin.

Respecting the nature of this special substance, the following further data are available:—

1. The substance in question is, as is well known, withdrawn from the sera by red blood-cells of the kind used for injection.

Experiment 5.—The serum of a rabbit, into whose peritoneal cavity red blood-cells derived from the hen had been injected was found, in the combination: *inactivated serum + erythrocytes of the hen + leucocytes of rabbit*, to bring about a very active phagocytosis of red blood-cells at 37° C. When 1 c.c. of the inactivated serum was added to the red blood-cells (previously washed with 0.85 per cent. sodium chloride solution in order to remove serum) of 0.25 c.c. of hen's blood, and the whole allowed to remain three hours at 0°, it was found that, after removal of the red blood-cells by centrifugalisation, the serum after the addition of erythrocytes and leucocytes as above, had lost its power of bringing about phagocytosis.

Experiment 6.—The serum of a rabbit, into whose peritoneal cavity red blood-cells derived from the calf had been injected, was found in the combination: *inactivated serum + erythrocytes of calf + leucocytes of rabbit*, to bring about phagocytosis at 37°. When the inactivated serum was treated with the red blood-cells of the calf, as in the preceding experiment, it was found subsequently to have lost the power of causing phagocytosis.

Experiments 7, 8, 9 were similarly carried out with guinea-pigs which received injections of the red blood-cells of the hen, calf, and rabbit respectively. In each case the inactivated serum employed, which was capable of bringing about very active phagocytosis *in vitro*, after treatment for three hours at a temperature of 15° C. with one-quarter of its volume of red blood-cells of the kind used for injection, was found to have lost its power of bringing about phagocytosis *in vitro*.

Thus the material, the existence of which in serum confers on the latter the property of exciting phagocytosis, combines with, or attaches itself to, the corresponding red blood-cells, and can therefore be withdrawn from the serum in the same way as amboceptor or agglutinin.

2. On the other hand, as the following experiments show, this material is not, in the same period of time, withdrawn from serum to the same extent by leucocytes.

Experiment 10.—To 0.3 c.c. of the serum employed in Experiment 6, leucocytes from the rabbit, occupying (after centrifugalisation) a volume of about 0.3 c.c., were added. After remaining at a temperature of 37° for three hours, the mixture was centrifuged and to a portion of the supernatant liquid, fresh leucocytes from the rabbit and red blood-cells from the hen, were added. At the end of an hour at 37° C., phagocytosis was found to have taken place, about 20 per cent. of the leucocytes present having ingested one to three red blood-cells.

Experiment 11.—The above experiment was repeated with the serum employed in Experiment 7, about 0.2 c.c. leucocytes being added to 0.2 c.c.

serum. On testing the phagocytic power of the serum by adding to it, at the end of three hours, leucocytes from the rabbit and red blood-cells from the calf, it was found that at the end of one hour, at 37°, about 25 per cent. of the leucocytes had taken up one to four red blood-cells.

Experiments 12 and 13.—These were similarly carried out with the serum of guinea-pigs, which had received intraperitoneal injections of red blood-cells of the calf and rabbit respectively. After the sera had been treated for three hours at 37° with an equal bulk of leucocytes from the guinea-pig, it was found on testing their phagocytic power that, at the end of an hour, 20 per cent. and 75 per cent. respectively of the leucocytes had taken up red blood-cells.

The rôle of the leucocytes, therefore, appears to be passive so long as the substance in question remains free in the serum, and only when a combination with red blood-cells has occurred do the leucocytes proceed to ingest the latter. Moreover, in experiments upon phagocytosis leucocytes other than those of the animal employed for injection of red blood-cells, may often be used. Thus it was found that when rabbits had been injected with red blood-cells from the hen or guinea-pig, their sera induced phagocytosis of red blood-cells of the kind injected, when leucocytes from the guinea-pig, goat, or sheep, instead of the rabbit, were used for experiment. In the same way with serum obtained from a goat injected with red blood-cells of the sheep, phagocytosis of the latter *in vitro* could be obtained with leucocytes from the rabbit, guinea-pig, sheep, or dove; and such examples can be multiplied.

3. When red blood-cells which have remained for some time in a serum capable of bringing about phagocytosis *in vitro*, are very thoroughly washed in 0.85 per cent. sodium chloride solution and then added to leucocytes suspended in saline solution, rapid phagocytosis may be obtained though the fluid employed is free from serum. It is essential in experiments of this kind that the serum used does not agglutinate the red blood-cells, for when the latter are heaped together phagocytosis cannot be satisfactorily observed. With the sera employed for Experiments 8 and 9, no agglutination occurred when red blood-cells from the calf and rabbit respectively were allowed to remain in four times their bulk of serum for one hour at 37°. After washing in saline solution and adding guinea-pig leucocytes, it was found that, at the end of 30 minutes at 37° C., 20 to 25 per cent. of the leucocytes had each taken up one to four red blood-cells. Still more rapid phagocytosis was obtained with red blood-cells of the calf and goat which had been similarly sensibilised with sera obtained from rabbits which had previously been several times injected with these varieties of red blood-cells.

4. The special constituent of serum which possesses the property of inducing phagocytosis is destroyed by heating.

Experiment 14.—The sera of three rabbits which were capable when diluted with four parts of saline solution of exciting active phagocytosis of the red blood-cells of the hen, calf, and goat respectively, were heated in the dilution of 1 in 5 for 30 minutes to 100° C. A slightly milky fluid free from precipitate was thus in each case obtained which, on testing, was found to have lost its power of causing phagocytosis. The sera of three guinea-pigs, injected with red blood-cells of the hen, calf, and rabbit respectively, were similarly found to have lost their power of causing phagocytosis, after 30 minutes exposure to 100°.

Experiment 15.—The six sera employed in the preceding experiment were heated, undiluted, to 69° for 30 minutes. All remained clear with the exception of two of the guinea-pig sera, which became slightly opaque and distinctly viscid. With these two no phagocytosis was obtainable; with the remaining sera phagocytosis was obtainable, but was not so vigorous as with unheated serum.

It appears, therefore, that while a temperature of 100° rapidly destroys the phagocytic action of serum, a temperature of 69° is much less effective.

5. The above characters serve to define the nature of the special constituent of serum which confers upon it the property of bringing about phagocytosis of red blood-cells, and show that its rôle is to prepare these cells for consumption by leucocytes. It is therefore a member of the group of opsonines first described by Wright and Douglas* in respect of bacteria.

In conclusion, it may be mentioned that erythrocytic opsonines are present in relatively small amount in normal sera. Thus, with the inactivated sera of the sheep, goat, dove, and hen, phagocytosis of the red blood-cells of the calf, goat, sheep, rabbit, and guinea-pig may, by suitably adjusting the conditions of experiment, be readily obtained. In the following experiment this is illustrated: in A the red blood-cells are insufficiently sensitised, so that no phagocytosis takes place, as is also the case in D, where the serum is apparently completely deopsinated; in F, on the other hand, owing to the relatively small amount of red blood-cells employed, the serum employed still retains sufficient opsonin to cause the appearance of phagocytosis when fresh red blood-cells are added.

* "The *Rôle* of the Blood Fluids in connection with Phagocytosis," 'Roy. Soc. Proc., 1904, vol. 72, p. 357; *cp.* "On the Nature of the Opsonic Action of the Blood Serum." W. Bulloch and E. E. Atkin, 'Roy. Soc. Proc.,' 1905, vol. 74, p. 379.

Experiment 16.—The following mixtures—

	A.	B.	C.
Inactivated serum of normal dove...	0·1 c.c.	0·1 c.c.	0·1 c.c.
Red blood-cells of guinea-pig	0·025 c.c.	0·005 c.c.	0·0015 c.c.

were kept (with constant shaking) at 30° C. for 2½ hours. To a loopful of A, B, and C respectively, a few leucocytes from the dove were added; at the end of one hour none of the leucocytes added to A contained red blood-cells, while 15 and 20 per cent. respectively of those added to B and C contained red blood-cells. A, B, and C were now centrifugalised, and to the cell free sera fresh red blood-cells of the guinea-pig and leucocytes of the dove added (D, E, F respectively); at the end of an hour, at 37° C., in D, none of the leucocytes contained red blood-cells, in E and F, 1 and 20 per cent. respectively of the leucocytes contained red blood-cells.
